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Rescue of recombinant Zika virus from a bacterial artificial chromosome cDNA clone

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DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY

UNIVERSITY of ROCHESTER MEDICAL CENTER

MEDICINE of THE HIGHEST ORDER

December 11th, 2018

Dear Editor,

We have submitted our manuscript **"Rescue of recombinant Zika virus from a bacterial artificial chromosome cDNA clone"** by Ávila-Pérez et al., which we would like to be considered for publication at Journal of Visualized Experiments (JoVE).

Like with other flaviviruses, the generation of Zika virus (ZIKV) full-length infectious cDNA clones has been hampered due to the toxicity of viral sequences during its amplification in bacteria. To overcome this problem, we have developed a non-traditional approach based on the use of a bacterial artificial chromosomes (BAC). Using this approach, the full-length cDNA copy of the ZIKV strain Rio Grande do Norte Natal (ZIKV-RGN) was generated from four synthetic DNA fragments and assembled into the single-copy pBeloBAC11 plasmid under the control of the human cytomegalovirus (CMV) immediate-early promoter. The assembled BAC cDNA clone was stable during propagation in bacteria and infectious recombinant (r)ZIKV was recovered in Vero cells after transfection of the BAC cDNA clone using Lipofectamine 2000. The protocol described here provides a powerful methodology for the generation of infectious clones of flaviviruses, including ZIKV, and other positive-strand RNA viruses, particularly those with large size genomes and stability problems during its propagation in bacteria.

Our approach to generate rZIKV presents the following advantages over previously described methods: i) The BAC plasmid pBeloBAC11, derived from the *E. coli* F-factor, allows the stable maintenance in bacteria of large and/or instable cDNAs due to its strictly controlled replication, leading to one or two copies per cell, that minimize the toxicity problem associated with several flavivirus sequences. ii) The manipulation and modification of the BAC cDNA clone is

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essentially similar to that of conventional plasmids with slight modifications due the large size of BAC cDNA clones and the presence of the plasmid in only one or two copies per cell. Notably, the BAC cDNA clone can also be modified into *E. coli* by homologous recombination using the Red recombination system. iii) The expression of ZIKV vRNA under the control of the RNA polymerase II-driven human CMV promoter[33] allows the intracellular expression of capped vRNA and the recovery of infectious virus without the need of an *in vitro* transcription step. iv) Infectious rZIKV is generated after direct transfection of susceptible cells (e.g. Vero) with the BAC cDNA clone. Due that DNA transfection in mammalian cells is more efficient than RNA transfection, the virus recovery efficiency with the BAC-approach is higher than observed using RNA transcripts, reducing the number of passages in culture cells to generate a viral stock and consequently, the introduction of undesired mutations by cell culture adaptation. Importantly, Vero cells are a cell line approved by the FDA for human vaccine production.

Thank you for your time and consideration.

I look forward to hearing from you.

Sincerely,

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TITLE:

Rescue of Recombinant Zika Virus from a Bacterial Artificial Chromosome cDNA Clone

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KEYWORDS:

Arbovirus, flavivirus, Zika virus, reverse genetics, full-length cDNA infectious clones, bacterial artificial chromosome, cDNA transfection, virus rescue

SUMMARY:

The recent epidemic of Zika virus highlights the importance of establishing reverse genetic approaches to develop vaccines and/or therapeutic strategies. Here, we describe the protocol to rescue an infectious recombinant Zika virus from a full-length cDNA clone assembled in a bacterial artificial chromosome under the control of the human cytomegalovirus immediate-early promoter.

ABSTRACT:

The association of Zika virus (ZIKV) infection with neurological complications during the recent worldwide outbreak and the lack of approved vaccines and/or antivirals have underscored the urgent need to develop ZIKV reverse genetic systems to facilitate the study of ZIKV biology and the development of therapeutic and/or prophylactic approaches. However, like with other flaviviruses, the generation of ZIKV full-length infectious cDNA clones has been hampered due to the toxicity of viral sequences during its amplification in bacteria. To overcome this problem, we have developed a nontraditional approach based on the use of bacterial artificial chromosomes (BACs). Using this approach, the full-length cDNA copy of the ZIKV strain Rio Grande do Norte Natal (ZIKV-RGN) is generated from four synthetic DNA fragments and assembled into the single-

copy pBeloBAC11 plasmid under the control of the human cytomegalovirus (CMV) immediate-early promoter. The assembled BAC cDNA clone is stable during propagation in bacteria, and infectious recombinant (r)ZIKV is recovered in Vero cells after transfection of the BAC cDNA clone. The protocol described here provides a powerful technique for the generation of infectious clones of flaviviruses, including ZIKV, and other positive-strand RNA viruses, particularly those with large genomes that have stability problems during bacterial propagation.

INTRODUCTION:

ZIKV is a mosquito-borne member of the Flavivirus genus within the *Flaviviridae* family that currently constitutes a global public health emergency[1]. Like other flaviviruses, ZIKV is an enveloped RNA virus with an icosahedral-like structure that contains a positive sense, single-stranded RNA molecule of about 10.8 kb (**Figure 1**)[2]. The viral genome encodes a large polyprotein of approximately 3,423 amino acids that is processed by viral and cellular proteases into three structural proteins (capsid [C], premembrane/membrane [prM/M], and envelope [E]) that are involved in the formation of the viral particles and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) that participate in genome replication, virus assembly, and evasion of the host immune response (**Figure 1**)[3].

Historically, ZIKV infection has been associated with a mild febrile illness[4,5]. However, the explosive recent pandemic of ZIKV infections throughout South and Central America, the South Pacific, and the Caribbean[6–8], and its association with the occurrence of Guillain-Barré syndrome and microcephaly[9–13], have changed the historic perception and potentiated the relevance of ZIKV as an important human pathogen. In this sense, the development of molecular tools, such as infectious cDNA clones, will facilitate the study of viral pathogenesis and the development of genetically defined vaccines and the identification of antiviral drugs for the treatment of ZIKV infection. As described for other flaviviruses, the generation of ZIKV infectious clones is difficult due to the presence of cryptic bacterial promoters in the viral genome[14] that allow the leaky expression of toxic viral proteins during the propagation of the cDNA clones in bacteria using standard high-copy-number plasmids[15–17]. To overcome this toxicity problem, several nontraditional approaches have been implemented successfully in the last two years[18]. These include the use of low-copy-number plasmids[19,20], the insertion of introns to disrupt the toxic regions[21–23], the in vitro ligation of cDNA fragments[24,25], mutational silencing of cryptic bacterial promoters present in the viral genome[26,27], infectious subgenomic amplicons (ISA)[28,29], the Gibson assembly method[30], and the use of circular polymerase extension reaction (CPER)[31].

Herein, we describe the detailed protocol for the engineering of a full-length cDNA clone of the ZIKV strain ZIKV-RGN[13], using a BAC to overcome the toxicity problem, and the rescue of infectious rZIKV by direct transfection of the BAC cDNA clone into Vero cells[32], a cell line approved by the Food and Drug Administration (FDA) for the development of human vaccines[33]. In this system, the full-length cDNA copy of the viral genome is assembled in the BAC plasmid pBeloBAC11[34] (**Figure 2A**), a low-copy-number plasmid (one to two copies per cell) derived from the *Escherichia coli* F-factor[35], which minimizes the toxicity of flavivirus sequences during its propagation in bacteria. The cDNA of the ZIKV genome is assembled in pBeloBAC11 under the control of the human CMV immediate-early promoter, to allow the expression of the viral (v)RNA

in the nucleus of transfected cells by cellular RNA polymerase II, and flanked at the 3'-end by the hepatitis delta virus (HDV) ribozyme (RZ), followed by the sequences of the bovine growth hormone (BGH) termination and polyadenylation signals to produce synthetic RNAs bearing authentic 5'- and 3'-ends of the viral genome, respectively (**Figure 2B**). This cDNA-launched system results in the intracellular expression of capped viral RNA, allowing the recovery of infectious ZIKV without the need for an in vitro transcription step. The BAC approach provides a powerful methodology applicable to constructing stable and fully functional infectious cDNA clones for other flaviviruses, as well as other positive-stranded RNA viruses[36–41].

PROTOCOL:

1. Construction of a ZIKV infectious cDNA clone in a BAC

NOTE: The strategy for the assembly of ZIKV in BACs is described for the RGN strain[13] (accession number KU527068) (**Figure 2**).

1.1. Select unique restriction sites appropriately spaced in the viral genome that are absent in the plasmid pBeloBAC11 (**Figure 2A**).

NOTE: For ZIKV-RGN, the restriction sites Pml I, Afe I, and BstB I (genomic positions 3,347, 5,969, and 9,127, respectively) were selected. In the case that no appropriate restriction sites are available in the viral genome, generate new restriction sites by introducing silent nucleotide mutations in the viral genome during the cDNA fragment design.

1.2. Generate by chemical synthesis four cDNA fragments spanning the full-length genome (Z1 to Z4), flanked by the CMV promoter at the 5'-end and the HDV RZ, the BGH termination, and polyadenylation sequences at the 3'-end (**Figure 2B**). Each fragment must be flanked by the selected restriction sites (step 1.1).

NOTE: Fragment Z1 is used as a backbone to clone the rest of the fragments in the pBeloBac11. For that end, it must contain the human CMV promoter and must be flanked at the 5'-end by ApaL I (to clone this fragment in pBeloBAC11) and Asc I (absent in the viral genome), and at the 3'-end by the restriction sites selected for the assembly of the infectious clone (Pml I, Afe I, and BstB I) followed by Mlu I (absent in the viral genome) and BamH I (to clone this fragment in pBeloBAC11). Fragment Z4 must contain the genomic region from the last restriction site selected (BstB I) to the end of the viral genome, followed by the HDV RZ, the BGH termination, and polyadenylation sequences, and the Mlu I restriction site (**Figure 2B**). Alternatively, the cDNA fragments (Z1–Z4) could be generated by a combination of standard reverse transcriptase polymerase chain reactions (RT-PCRs) and overlapping PCRs using specific oligonucleotides.

1.3. Assemble the infectious cDNA clone by sequential cloning of fragments Z1 to Z4 into pBeloBAC11 (**Figure 2B**).

1.3.1. Digest the pBeloBAC11 plasmid and the Z1 fragment with ApaL I and BamH I. For that, mix

2 µg of pBeloBAC11 plasmid or 1 µg of Z1 fragment with 10 µL of 10x reaction buffer, 20 units of each enzyme, and water to reach a final volume of 100 µL. Incubate at 37 °C for 2 h.

1.3.2. Dephosphorylate the BAC vector using shrimp alkaline phosphatase (SAP). To that end, add 2.5 µL (2.5 units) of SAP to the digested BAC and incubate at 37 °C for 1 h. Heat-inactivate the SAP by incubation at 75 °C for 15 min.

1.3.3. Purify the dephosphorylated BAC vector and fragment Z1 by agarose gel electrophoresis using a commercial gel clean-up kit optimized for the purification of DNA fragments larger than 10 kb (see the **Table of Materials**).

1.3.4. Perform the ligation reaction to generate the plasmid (p)BAC-Z1. To that end, mix 150 ng of purified digested BAC vector with the purified insert using a molar ratio of vector-to-insert of 1:3, 1.5 µL of 10x T4 DNA ligase buffer containing 10 mM ATP, one unit of T4 DNA ligase, and water to a final volume of 15 µL.

1.3.5. Incubate the ligation mixture for 20 h at 16 °C. As control of the ligation reaction, perform in parallel a ligation reaction without insert. Heat-inactivate the ligase by incubation at 65 °C for 15 min.

NOTE: In the case of blunt-ended DNAs, incubate the ligation reaction for 20 h at 14 °C. Due to the large size of the BAC vector (**Figure 2A**), it is essential to use larger amounts of vector and insert than ligations, using conventional plasmids in order to increase the ligation efficiency.

1.3.6. Transform 50 µL of *E. coli* DH10B electrocompetent cells with 2 µL of the ligation reaction (step 1.3.5) by electroporation (25 µF capacitance, 2.5 kV, and 100 Ω resistance), using electroporation cuvettes fitted with electrodes spaced at intervals of 0.2 cm, following standard protocols.

1.3.7. Transfer the cells to a polypropylene tube with 1 mL of SOC medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose [pH 7.0]) and incubate them at 37 °C for 1 h with gentle shaking (200–250 rpm). Plate the cells onto Luria broth (LB) agar plates containing 12.5 µg/mL chloramphenicol and incubate them at 37 °C for 16 h.

1.3.8. Pick 8 to 12 bacterial colonies, make a replica on a fresh LB agar plate containing 12.5 µg/mL chloramphenicol, and test whether they contain the correct insert by direct PCR analysis using specific oligonucleotides.

1.3.9. Pick a positive colony from the replica plate, grow it in 100 mL of LB containing 12.5 µg/mL of chloramphenicol, and isolate the BAC cDNA by the alkaline lysis method using a commercial plasmid midi kit, following the recommendation for the purification of large low-copy plasmids (see the **Table of Materials**).

NOTE: BAC cDNA prepared by this method can be contaminated with up to 30% of bacterial genomic DNA, but it is suitable in quality to perform restriction analysis, sequencing, and cloning. Depending on the BAC size, yields of 4–6 µg of the BAC plasmid can be obtained.

1.3.10. Verify the genetic integrity of the cloned cDNA by restriction analysis. Digest 500 ng of the pBAC-Z1 plasmid with Asc I and Pml I at 37 °C for 1 h and confirm the presence of the Z1 fragment by gel electrophoresis. To further confirm that unwanted mutations have not been introduced, sequence the insert with specific oligonucleotides.

1.3.11. Starting from the plasmid pBAC-Z1 containing the selected restriction sites (*Pml* I, *Afe* I, *BstB* I, and Mlu I), sequentially clone fragments Z2 to Z4 to generate the full-length infectious cDNA clone pBAC-ZIKV (**Figure 2B**), following similar experimental approaches as described above for fragment Z1 (steps 1.3.1–1.3.10).

2. Preparation of high-purity pBAC-ZIKV for the rescue of infectious rZIKV

NOTE: The large-scale preparation of an ultrapure pBAC-ZIKV infectious clone, suitable for the transfection and rescue of infectious viruses, is performed by alkaline lysis with a commercial kit specifically developed for BAC purification (see the **Table of Materials**). The kit must include an ATP-dependent exonuclease digestion step that removes bacterial genomic DNA contamination, allowing the isolation of BAC cDNA with a grade of purity similar to that obtained with the cesium chloride method.

2.1. Grow a single colony of *E. coli* DH10B carrying the pBAC-ZIKV infectious clone in 5 mL of LB medium containing 12.5 µg/mL of chloramphenicol at 37 °C for 8 h with gentle shaking (200–250 rpm).

2.2. Add 1 mL of bacterial culture (step 2.1) to 500 mL of LB with 12.5 µg/mL of chloramphenicol in a 2 L flask and grow the cells at 37 °C for 14–16 h (till an OD_{600} of 0.6–0.8).

NOTE: Rich broths, such as Terrific Broth (TB), can produce extremely high cell densities, resulting in a lower yield and less purity of the BAC cDNA.

2.3. Purify the BAC infectious cDNA clone by alkaline lysis using a commercial kit specifically developed for BAC purification, following the manufacturer's specifications (see the **Table of Materials**). Keep the purified BAC cDNA at 4 °C. Depending on the BAC size, yields of 30 µg of ultrapure BAC cDNA clone can be obtained.

NOTE: Do not dry the DNA pellet for more than 5 min, as overdrying will make it difficult to dissolve the BAC cDNA. Due to the large size of the BAC cDNA clone, avoid vortexing or pipetting to prevent plasmid shearing.

3. Rescue of infectious rZIKV from the BAC cDNA clone by transfection of Vero cells

NOTE: Infectious rZIKV is recovered by the transfection of Vero cells with the pBAC-ZIKV BAC cDNA clone, using a commercial cationic lipid transfection reagent (see the **Table of Materials**; **Figure 3**).

3.1. One day before transfection, plate on 6-well plates 5 x 10^5 Vero cells/well in growth medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with 5% fetal bovine serum [FBS], 2 mM L-glutamine, and 1% nonessential amino acids) without antibiotics to raise 90% confluent cell monolayers by the time of transfection.

NOTE: We recommend performing the virus rescues in triplicates.

3.2. Equilibrate serum-reduced medium (see the **Table of Materials**) at room temperature and prepare transfection mixtures in sterile microfuge tubes for each transfection sample as follows.

3.2.1. Add 4 µg of the BAC cDNA clone in 250 µL of serum-reduced medium and mix carefully, avoiding vortexing to prevent plasmid shearing.

3.2.2. In a separate tube, dilute 12 µL of transfection reagent (1 mg/mL) (see the **Table of Materials**) in 250 µL of serum-reduced medium, mix by vortexing, and incubate at room temperature for 5 min.

3.2.3. Combine the diluted BAC cDNA and the transfection reagent (with a 1:3 ratio of DNA:transfection reagent), mix carefully while avoiding vortexing, and incubate at room temperature for 20–30 min.

3.3. During the incubation period of the BAC cDNA/transfection reagent mixture, wash Vero cells with growth medium without antibiotics and leave the cells in 1 mL of fresh medium without antibiotics.

NOTE: The addition of antibiotics during the transfection process may decrease transfection efficiency.

3.4. Distribute dropwise the 500 µL of the BAC cDNA/transfection reagent mixture (of step 3.2.3) onto the Vero cells, mix them by rocking the plate back and forth, and incubate the cells in a 5% CO_2 humidified incubator at 37 °C for 6 h.

3.5. Remove the transfection medium, add 2 mL of fresh growth medium with antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin), incubate the cells in a 5% CO_2 humidified incubator at 37 °C, and check every day for the induction of cytopathic effect (CPE).

NOTE: ZIKV CPE is quite pronounced in Vero cells and it is characterized by the presence of rounded and birefringent cells that detach and float in the tissue culture supernatant.

3.6. Collect aliquots (100 µL) of the Vero cell tissue-culture supernatants (step 3.5) every 24 h to

determine the efficiency of virus recovery by assessing the presence of ZIKV using a standard plaque assay on fresh Vero cells (**Figure 4A**).

3.7. After four to six days of transfection, when the CPE is around 50%–75% (**Figure 4B**), collect the tissue culture supernatants in 15 mL conical tubes and centrifuge at 2,000 x *g* for 10 min at 4 °C to remove cellular debris.

3.8. Harvest the supernatants containing rZIKV and discard the cell pellets. Aliquot the supernatant in cryotubes and store them at -80 °C to further confirm the presence of rescued rZIKV.

4. Titration of recovered rZIKV

4.1. A day before titration, seed on 12-well plates 2.5 x 10^5 Vero cells/well in growth medium to raise 90% confluent cell monolayers by the time of titration.

NOTE: We recommend conducting the titration of the recovered rZIKV in triplicates.

4.2. Remove an aliquot of the supernatant from the freezer (step 3.8) and make serial 10-fold dilutions in growth medium without FBS.

4.3. Wash Vero cells 2x with phosphate-buffered saline (PBS) and add 200 µL/well of each virus dilution in triplicate. Place plates in a 5% CO_2 humidified incubator at 37 °C and rock every 15 min for a 90 min adsorption period.

4.4. Remove the viral inoculum, overlay the cells with 2 mL of growth medium containing 2% FBS, 1% DEAE-dextran, and 0.6% Agar Noble, and incubate at 37 °C under 5% CO_2 for 3–4 days.

NOTE: It is possible to use agarose, methylcellulose, or other overlay media. The time for proper plaque formation will vary depending on the overlay used and the ZIKV strain.

4.5. Fix ZIKV-infected cells with 1 mL/well of 4% formaldehyde diluted in PBS at room temperature for 1 h, remove the overlay, and visualize the viral plaques by staining them with 0.1% crystal violet in 20% methanol at room temperature for 15 min.

4.5.1. Discard the crystal violet, wash 3x with water, allow the plates to dry, and manually count the plaques (**Figure 4C**, left panel). Viral titer is calculated as plaque forming units per milliliter (PFU/ml).

4.6. Alternatively, viral plaques can be visualized by immunostaining with the pan-flavivirus E protein mouse monoclonal antibody (mAb) 4G2 (**Figure 4C**, right panel).

4.6.1. To evaluate ZIKV plaques by immunostaining, after the fixing and removal of the overlay agar as described above (in step 4.5), wash the cells 2x with PBS and permeabilize them by

incubation with 200 µL/well of 0.5% Triton-X100 in PBS for 15 min at room temperature.

4.6.2. Remove the permeabilization solution, wash the cells 3x with PBS, and block them with 200 µL/well of blocking solution (10% FBS in PBS) for 1 h at room temperature.

NOTE: Other standard blocking solutions (e.g., 2.5% BSA in PBS) can be used in this step.

4.6.3. Remove the blocking solution and incubate the cells with 200 µL/well of the pan-flavivirus E protein mAb 4G2 diluted in blocking solution (1 µg/mL) for 1 h at 37 °C.

NOTE: Other mAb or polyclonal antibodies (pAb) can be used instead of 4G2 for the immunostaining and detection of viral plaques.

4.6.4. Wash the cells 3x with PBS, and incubate them with 200 µL of biotinylated anti-mouse secondary antibody diluted (following the manufacturer's recommendation) in blocking solution for 1 h at 37 °C.

4.6.5. Remove the secondary antibody, wash the cells 4x with PBS, and visualize the viral plaques using an avidin/biotin-based peroxidase kit following the manufacturer's specifications (see the **Table of Materials**).

5. Confirmation of successful rZIKV rescue

NOTE: To further confirm the identity of the rescued virus, ZIKV E protein expression is analyzed by immunofluorescence using the mouse mAb 1176-56 specific for ZIKV E protein (**Figure 4D**). This mAb is specific for ZIKV E protein, contrary to the situation of the pan-flavivirus E protein mAb 4G2 (step 4.6.3). Alternatively, the virus identity can be confirmed by sequencing.

5.1. A day before immunofluorescence analysis, seed coverslips in 24-well plates containing 1 x 10^5 Vero cells/well in growth medium to raise 90% confluent cell monolayers by the time of infection.

5.2. Wash the cells 2x with PBS and infect them with a multiplicity of infection (MOI) of 0.5 PFU/cell of the rescued virus in growth medium without FBS (100 µL/well) in triplicate. Incubate the plates at 37 °C for 90 min.

5.3. After viral adsorption, remove the viral inoculum, add 0.5 mL of fresh growth medium with 2% FBS, and incubate the cells in a 5% CO_2 humidified incubator at 37 °C for 48 h.

5.4. Remove the tissue culture supernatant, fix the cells with 150 µL/well of 4% paraformaldehyde in PBS for 20 min at room temperature, and permeabilize the cells with 150 µL/well of 0.5% Triton-X100 in PBS for 15 min at room temperature.

5.5. After removing the permeabilization solution and washing the cells 3x with PBS, block the

cells with 150 µL/well of blocking solution during 1 h at room temperature.

NOTE: Cells can be blocked with alternative blocking solutions (e.g., 2.5% BSA in PBS).

5.6. Remove the blocking solution and incubate the cells with 100 µL/well of mAb 1176-56, specific for ZIKV E protein, diluted in blocking solution (1 µg/mL) for 1 h at 37 °C.

5.7. Wash the cells 3x with PBS, incubate them at room temperature for 1 h with 100 µL/well of Alexa Fluor 488-conjugated anti-mouse secondary antibody diluted (following the manufacturer's recommendation) in blocking solution, wash the cells extensively with PBS, and incubate them with 150 µL/well of 4',6'-diamidino-2-phenylindole (DAPI; 1 mg/mL) diluted 1:200 in PBS at room temperature for 10 min.

5.8. Wash the cells 3x with PBS, mount the coverslips on an antifade mounting medium (see the **Table of Materials**), and analyze the samples under a fluorescence microscope.

NOTE: For long-term storage, store samples at 4 °C, protected from light.

6. Amplification and generation of viral stocks

NOTE: Once the identity of the rescued virus is confirmed (section 5), amplify the virus on Vero cells and generate viral stocks for further studies.

6.1. Grow Vero cells in 100 mm x 21 mm plates at 90% confluence and infect them with an MOI of 0.1 PFU/cell as described before.

6.2. When the CPE is around 75% (approximately 48–72 h postinfection), collect the tissue culture supernatant in a 50 mL conical tube and centrifuge at 2,000 x *g* for 10 min at 4 °C to remove the cellular debris.

6.3. Harvest the supernatant containing rZIKV and discard the cell pellet. Aliquot the supernatant in cryotubes and store them at -80 °C.

6.4. Remove a virus aliquot from the freezer and determine the viral titer by plaque assay as described before (section 4).

REPRESENTATIVE RESULTS:

The protocol described here allows for the generation of stable ZIKV full-length cDNA infectious clones using a BAC to minimize the toxicity problems associated with several flaviviral sequences. Efficient recovery of infectious rZIKV from the BAC cDNA clone can be easily achieved after the transfection of susceptible Vero cells (**Figure 2**). Using this protocol, we have generated a stable full-length cDNA clone of the ZIKV strain RGN[32] by sequentially cloning four overlapping cDNA fragments into the BAC plasmid pBeloBAC11[34] using conventional cloning methods and unique restriction sites present in the viral genome (**Figure 2**). The full-length cDNA clone was flanked at

the 5'-end by the human CMV promoter to allow the expression of vRNA in the nucleus of transfected cells, and at the 3'-end by the HDV RZ followed by the BGH polyadenylation and termination sequences, to produce RNAs containing the exact 3'-end of the viral genome (**Figure 2**). The stability in bacteria of the generated BAC cDNA clone, together with its easy manipulation using standard recombinant DNA technologies, highlights the potential of the BAC cDNA approach for the rapid and reliable generation of stable full-length cDNA clones of ZIKV and other flaviviruses or positive-stranded RNA viruses with unstable viral genomes.

Once the BAC cDNA clone was assembled (**Figure 2**), infectious virus could be easily recovered after the direct transfection of susceptible Vero cells with the BAC cDNA clone using cationic liposomes (**Figure 3**). This cDNA-launched system allowed the intracellular expression of the capped *vRNA*, allowing the recovery of infectious viruses without the need for an in vitro transcription step. Using this approach, we were able to rescue rZIKV-RGN with titers higher than 10^6 PFU/mL at 5 days posttransfection (**Figure 4A**). In addition, the rescued virus induced a clear CPE (**Figure 4B**), generated homogeneous plaques of about 2 mm in size (**Figure 4C**), and its identity was confirmed by sequencing (data not shown) and immunofluorescence analysis using the mAb specific for ZIKV E protein, 1176-56 (**Figure 4D**). In vitro data indicate that the recovered rZIKV-RGN replicated efficiently in Vero cells and to levels compared to a natural ZIKV isolate[32] (data not shown). Overall, these results demonstrate that infectious rZIKV can be rescued from full-length cDNA clones assembled in a BAC.

FIGURE AND TABLE LEGENDS:

Figure 1: ZIKV genome organization and virion structure. (**A**) Genome organization: ZIKV contains a positive single-stranded RNA that is translated as a single polyprotein. The translated polyprotein was cleaved by viral (arrows) and host (diamonds) proteases to produce the structural proteins capsid (C, blue), matrix (M, brown), and envelop (E, green), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The 5' and 3' untranslated regions (UTRs) at the end of the viral genome are indicated with black lines. (**B**) Virion structure: ZIKV virions were decorated with the E and M proteins, anchored in a lipid bilayer with an icosahedral-like structure. Under the viral envelop was the viral nucleocapsid composed of the C protein associated with the viral genomic RNA. This figure has been adapted from Ávila-Pérez et al.[18].

Figure 2: Assembly of the ZIKV full-length infectious cDNA clone in a BAC. (**A**) Schematic representation of the pBeloBAC11 BAC: The regulatory genes *parA, parB*, *parC*, and *repE*, the F-factor replication origin (*OriS*), the chloramphenicol resistant gene (Cm^r), and the *lac*Z gene are indicated. The relevant restriction sites used to assemble the infectious ZIKV cDNA clone are underlined. (**B**) Assembly of ZIKV full-length infectious cDNA clone into the pBeloBAC11 BAC: Four overlapping DNA fragments (Z1–Z4), covering the entire ZIKV genome (**Figure 1**) and flanked by the indicated restriction sites, were generated by chemical synthesis and sequentially cloned into pBeloBAC11 to generate the infectious ZIKV cDNA clone pBAC-ZIKV. The full-length ZIKV infectious cDNA was assembled under the control of the human cytomegalovirus immediate-early promoter (CMV) and flanked at the 3'-end by the HDV ribozyme (RZ) and the bovine growth

hormone (BGH) termination and polyadenylation sequences. The acronyms for the viral genes and regulatory elements are as described in **Figure 1**.

Figure 3: Workflow to generate rZIKV from the BAC cDNA clone. Vero cells at 90% of confluence were transfected in monolayer with the ZIKV full-length infectious cDNA clone pBAC-ZIKV (**Figure 2**) using cationic liposomes. At 4–6 days posttransfection, when CPE was evident, tissue culture supernatants containing rZIKV were collected and evaluated for the presence of virus (**Figure 4**) and used for viral amplification in Vero cells.

Figure 4: Recovery and in vitro characterization of rZIKV. (**A**) Rescue of infectious rZIKV from the BAC cDNA clone: Vero cells at 90% of confluence (6-well plate format, triplicates) were mock-transfected or transfected with 4 µg/well of pBAC-ZIKV (**Figure 3**), and at the indicated days posttransfection, virus titers in tissue culture supernatants were determined by plaque assay (PFU/mL). The error bars indicate standard deviations from three different transfection experiments. The dotted black line indicates the limit of detection (50 PFU/mL). (**B**) Viral CPE: Vero cells at 90% confluence (6-well plate format, triplicates) were mock-infected (top) or infected (MOI of 0.5 PFU/cell) with rZIKV, and at 48 h postinfection, the presence of CPE was evaluated by light microscopy. The scale bars = 100 µm. (**C**) Viral plaque assay and immunostaining: Vero cells at 90% confluence (12-well plate format) were infected with rZIKV, and at 72 h postinfection, viral plaques were visualized by crystal violet staining (left) or by immunostaining (right) using the pan-flavivirus E protein mAb 4G2. The scale bars = 5 mm. (**D**) Immunofluorescence: Vero cells at 90% confluence (24-well plate format, triplicates) were infected (MOI of 0.5 PFU/cell) with rZIKV and, at 48 h postinfection, analyzed by immunofluorescence using the mAb 1176-56, specific for ZIKV E protein. Cell nuclei were stained with DAPI. A representative merged image of ZIKV-infected Vero cells is shown. The white square in the top right represents an enlarged image of ZIKV-infected Vero cells. The scale bars = 100 µm.

DISCUSSION:

Infectious cDNA clones constitute essential molecular tools for basic research of RNA viruses and the development of vaccines and/or the identification of antiviral strategies. However, for many positive-stranded RNA viruses, including flaviviruses, the generation of infectious cDNA clones are difficult due to the instability of the cloned cDNAs when propagated in bacteria using standard high-copy-number plasmids. In the case of ZIKV and other flaviviruses, this instability is mainly due to the leaky expression of toxic viral proteins from cryptic bacterial promoters present in the viral genome[14–17]. Here, we describe an alternative and powerful protocol to generate a stable ZIKV full-length infectious cDNA clone as a single plasmid, based on the use of the BAC plasmid pBeloBAC11[34] (**Figure 2A**) to overcome the toxicity problem, the use of the CMV promoter to allow the expression of the vRNA in the nucleus of transfected cells, and the HDV RZ to generate vRNAs with accurate 3'-ends (**Figure 2B**). Using this method, we have successfully generated a fully stable infectious clone of the ZIKV strain RGN that allows the efficient and reliable recovery of infectious rZIKV after the direct transfection of susceptible Vero cells (**Figure 3** and **Figure 4**).

A huge effort has been made in the last few years to overcome the instability problems associated with ZIKV infectious cDNA clones, and several approaches have been successfully implemented[18], including the in vitro ligation of cDNA fragments[24,25], low-copy plasmids[19,20], the inactivation of cryptic bacterial promoters by the introduction of silent mutations[26,27], intron insertion[21–23], the Gibson assembly method[30], the ISA method[28,29], and the use of CPER[31]. Although these approaches overcome the toxicity problem and are useful to generate ZIKV infectious cDNA clones, some of them are laborious and present several disadvantages, including the need for in vitro ligation and transcription steps that reduce virus recovery efficiency or the introduction of a high number of silent mutations to inactivate cryptic bacterial promoter that could affect viral fitness, among others. The approach described in this protocol presents the following advantages. i) The BAC plasmid pBeloBAC11[34] has a strictly controlled replication, keeping one or two copies of plasmid per cell, which minimizes toxicity and allows stable maintenance in bacteria of instable cDNAs. ii) The propagation and modification of BAC plasmids are almost similar to those of conventional plasmids, considering the slight modifications described in this protocol to manipulate large-size BAC-DNA fragments and low-copy plasmids. Notably, the BAC cDNA clone can also be modified into *E. coli* by homologous recombination using the Red recombination system[42–44]. iii) The use of CMV promoter allows the intracellular expression of capped ZIKV vRNA and the recovery of infectious viruses without requiring an in vitro transcription step. iv) Infectious rZIKV is generated after the direct transfection of susceptible cells (e.g., Vero) with the BAC cDNA clone. Since DNA transfection in mammalian cells is more efficient than RNA transfection, the virus recovery efficiency with the BAC approach is higher than that observed using RNA transcripts, reducing the number of passages in culture cells to generate a viral stock and, consequently, limiting the introduction of unwanted mutations by cell culture adaptation.

Finally, the potential of the BAC approach is supported by the successful use of this method (with slight modifications) to engineer infectious cDNA clones of other flaviviruses, including dengue virus[36], and several coronaviruses of high impact in human and animal health, such as transmissible gastroenteritis coronavirus[37] (TGEV), feline infectious peritonitis virus[38] (FIPV), human coronavirus OC43[39] (HCoV-OC43), severe acute respiratory syndrome coronavirus[40] (SARS-CoV), and Middle East respiratory syndrome coronavirus[41] (MERS-CoV), among others.

In the protocol described here, there are two critical steps that should be considered. One important consideration is identifying appropriate unique restriction sites in the viral genome that are absent in the BAC plasmid. If no adequate restriction sites are available, new restriction sites can be generated during the cloning design by the introduction of silent nucleotide mutations. Another important issue is that the BAC plasmids are present in only one or two copies per cell, and therefore, low yields of BAC plasmids with a high contamination of bacterial genomic DNA are obtained using standard protocols designed for high- and medium-copy-number plasmids. This potential problem is easily overcome using large culture volumes and purifying the BAC plasmid with a commercial kit specifically developed for BAC purification.

In summary, we have developed a powerful ZIKV reverse genetic approach based on the use of a BAC that could be adapted to generate stable and fully functional infectious cDNA clones of other positive-stranded RNA viruses to facilitate the study of the biology of these viruses and the

development of vaccines and/or to facilitate the identification of antiviral drugs.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

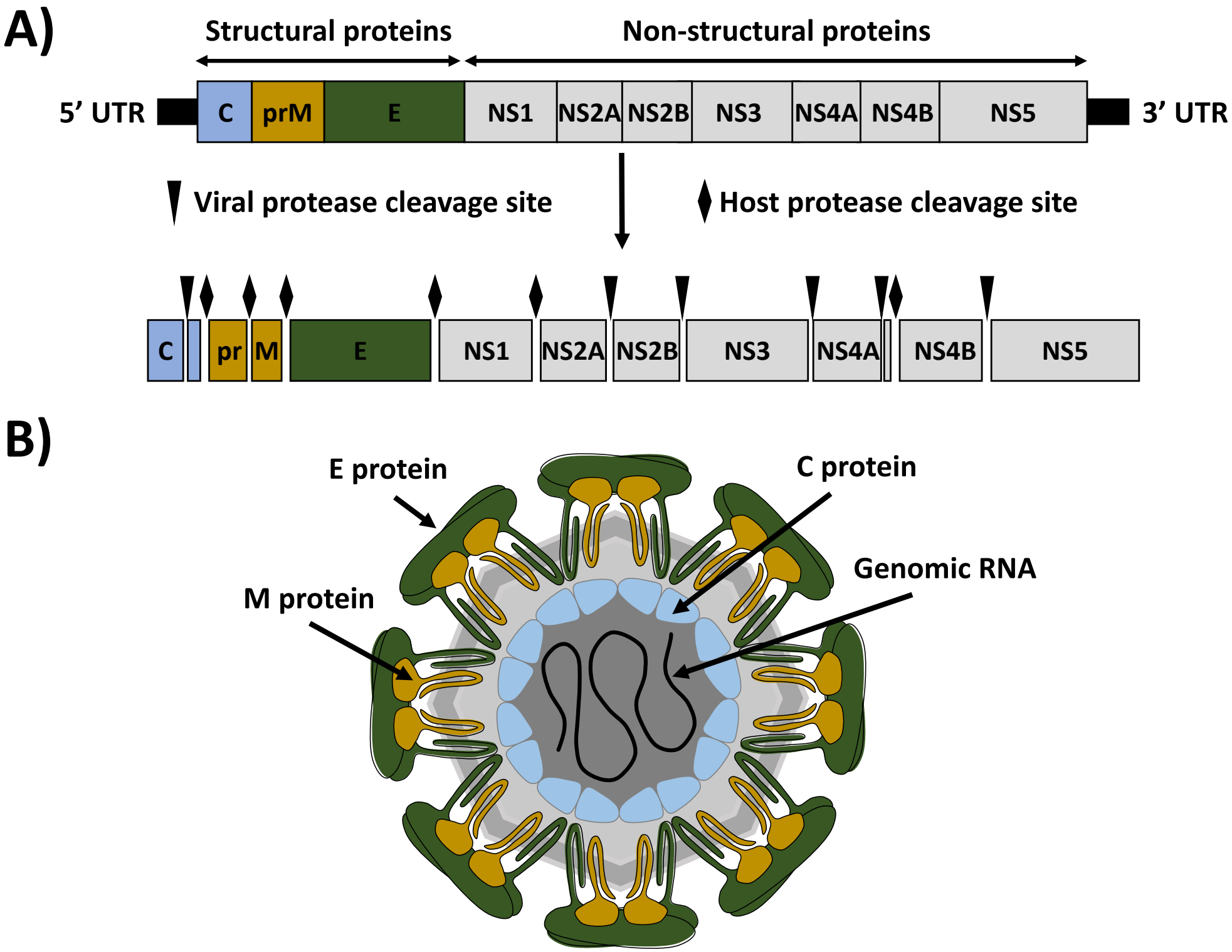

Figure 2

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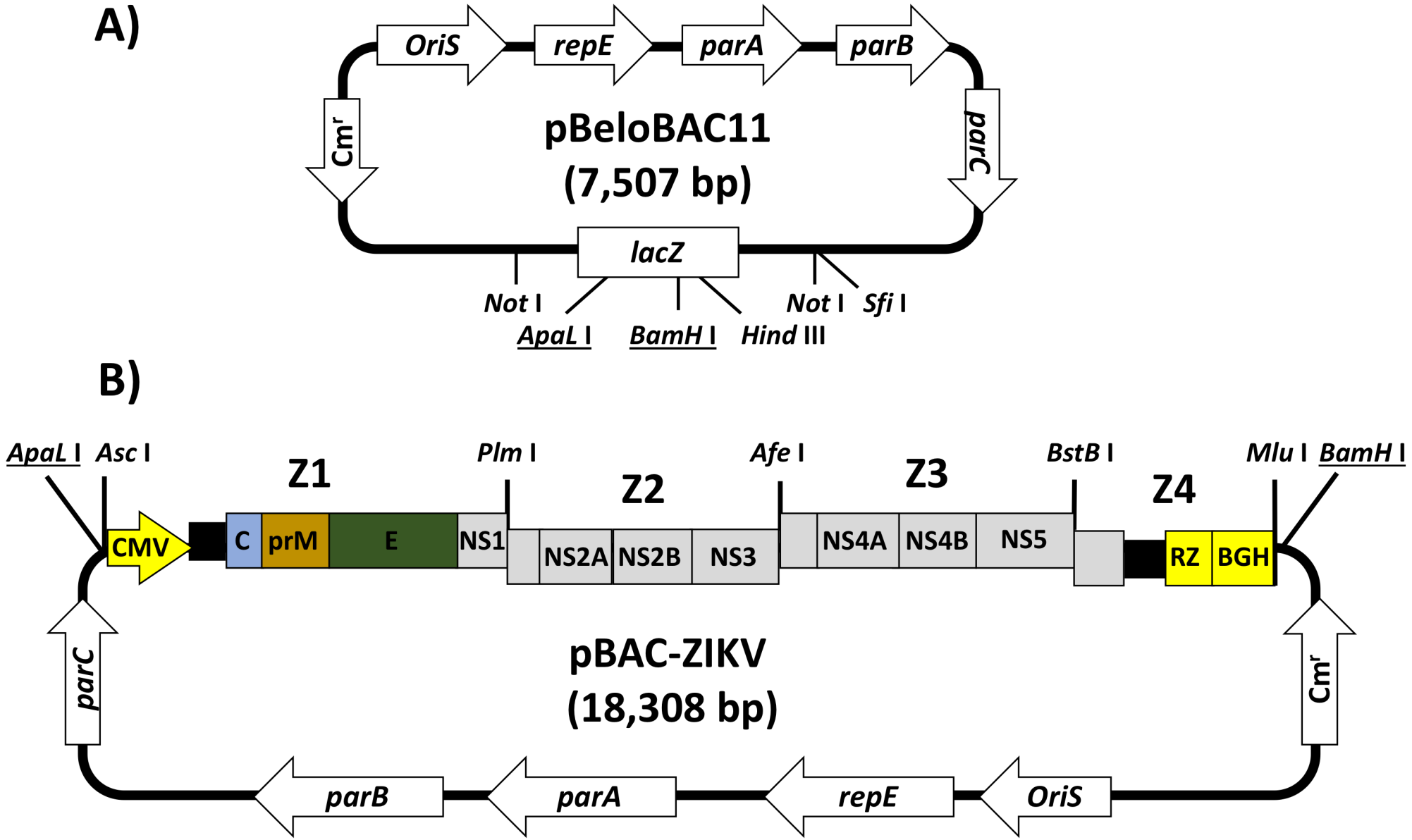
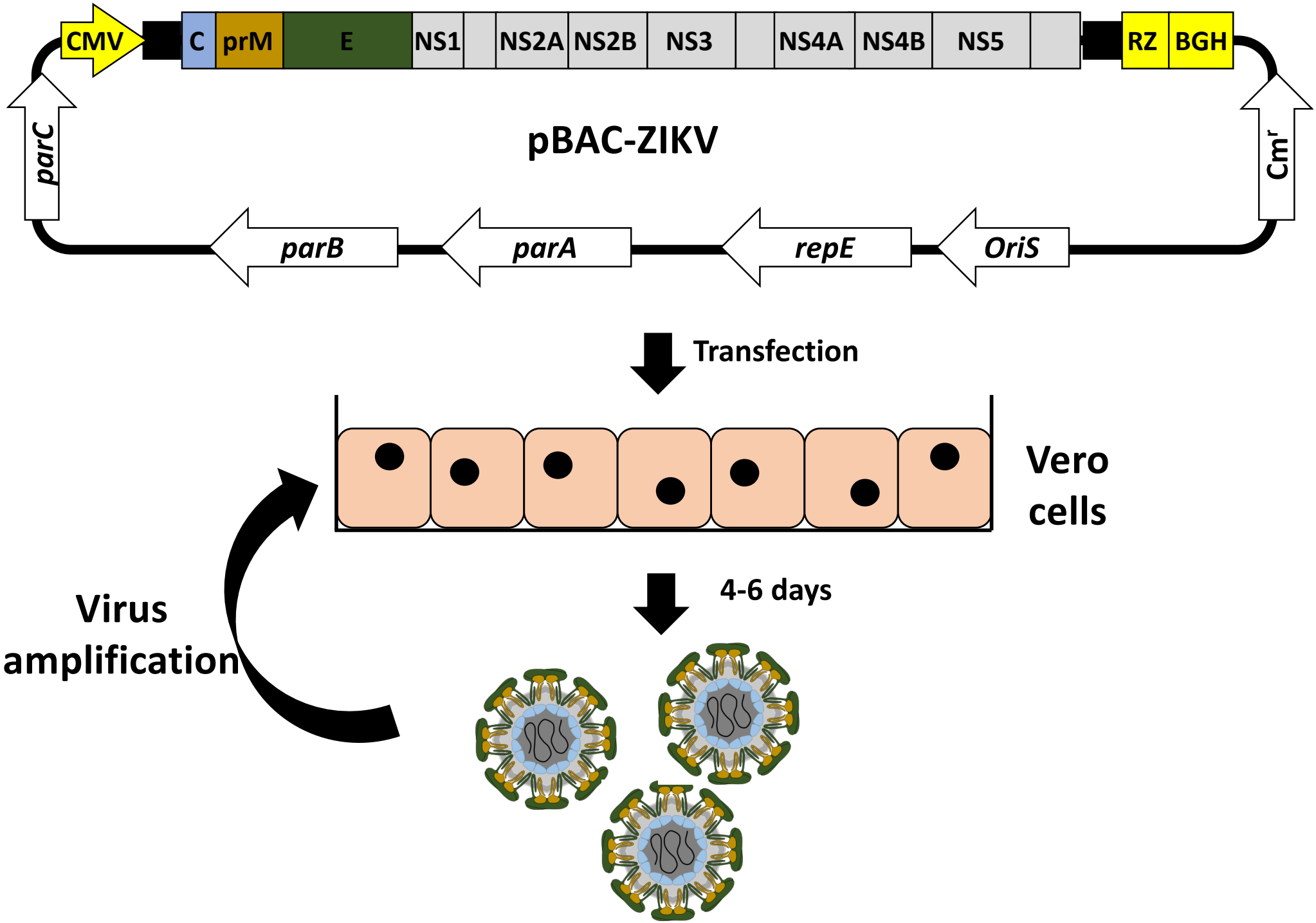


Figure 3



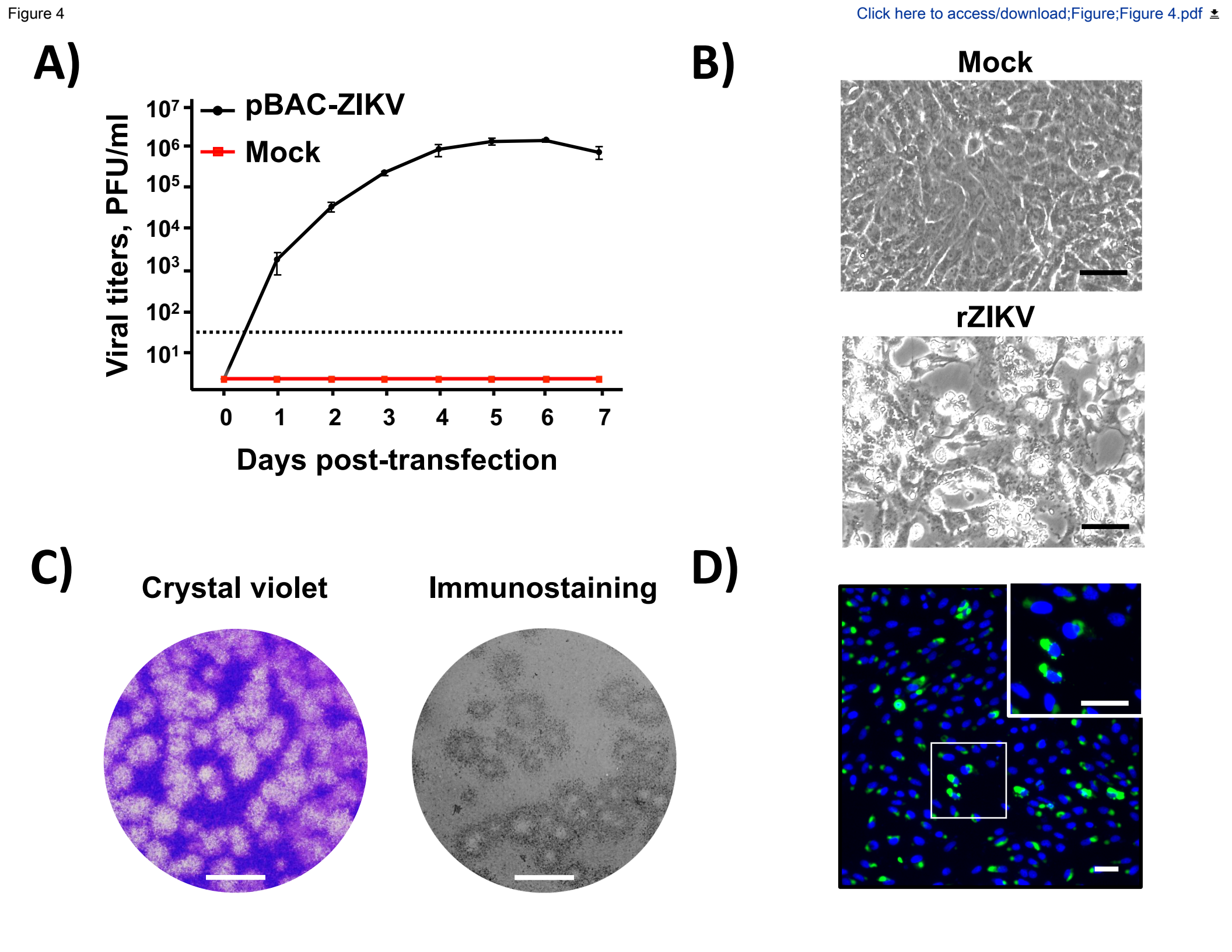


Table of materials

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Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
1. Molecular Biology Reagents			
Afe I	New England BioLabs	R0652S	10,000 units/mL
AmpliTaq DNA Polymerase	ThermoFisher Scientific (Applied Biosystems)	N8080161	5,000 Units/mL
ApaL I	New England BioLabs	R0507S	10,000 units/mL
Asc I	New England BioLabs	R0558S	10,000 units/mL
BamH I	New England BioLabs	R0136S	10,000 units/mL
BstB I	New England BioLabs	R0519S	20,000 units/mL
Chloramphenicol	Sigma-Aldrich	C0378	
ElectroMAX DH10B Cells	ThermoFisher Scientific (Invitrogen)	18290015	Electocompetent DH10B cells
Electroporation Cuvettes, 0.2 cm	Bio-Rad	165-2086	
Ethanol	Merck	100983	Flamable
Isopropanol	Merck	109634	Flamable
Large-Construct Kit (10)	QIAGEN	12462	For high-purity BAC preparation
LB Broth	ThermoFisher Scientific (Invitrogen)	12780029	Can be homemade as well
LB with Agar	ThermoFisher Scientific (Invitrogen)	22700041	Can be homemade as well
Methanol	Merck	106009	Flamable
Mlu I	New England BioLabs	R0198S	10,000 units/mL
Oligonucleotides	IDT	N/A	
Plasmid pBeloBAC11	New England BioLabs	ER2420S (E4154S)	
Plasmid Midi Kit (25)	QIAGEN	12143	For midle-scale preparation of BAC plasmids
Pml I	New England BioLabs	R0532S	20,000 units/mL
Polypropylene tubes (10 mL)	DeltaLab	175724	Other commercial sources are acceptable
QIAEX II Gel Extraction Kit (150)	QIAGEN	20021	Gel-clean-up kit optimized for DNA fragments larger than 10
Shrimp AlKaline Phosphatase (rSAP)	New England BioLabs	M0371S	1,000 units/mL
SOC Medium	ThermoFisher Scientific (Invitrogen)	15544034	Can be homemade as well
Synthesis of cDNA fragments	Bio Basic	N/A	
T4 DNA Ligase	Sigma-Aldrich (Roche)	10481220001	1,000 units/mL
2. Cell Culture Reagents			
6-Well Plates	ThermoFisher Scientific (Nunc)	140675	
12-Well Plates	ThermoFisher Scientific (Nunc)	150628	
24-Well Plates	ThermoFisher Scientific (Nunc)	142485	
Agar Noble	VWR	214230	
Alexa Fluor 488 Conjugate ant-mouse secondary antibody	Varies	N/A	
Biotinylated Anti-Mouse Secondary Antibody	Varies	N/A	
Cell Culture Dishes (100x21 mm)	ThermoFisher Scientific (Nunc)	172931	
Conical Tubes (15 mL)	VWR	525-0150	
Conical Tubes (50 mL)	VWR	525-0155	
Crystal Violet	Sigma-Aldrich	C6158	
DAPI	Sigma-Aldrich	D9542	Toxic and carcinogenic
DEAE-Dextran	Sigma-Aldrich	D9885	
DMEM	ThermoFisher Scientific (Gibco)	11995065	
Fetal Bovine Serum (FBS)	ThermoFisher Scientific (HyClone))	SV30160.03	
Formaldehyde	Sigma-Aldrich	F8775	Toxic and carcinogenic
L-Glutamine	ThermoFisher Scientific (Gibco)	25030081	
Lipofectamine 2000	ThermoFisher Scientific (Invitrogen)	11668019	Transfection reagent
Nonessential amino acids	ThermoFisher Scientific (Gibco)	11140035	
Opti-MEM I Reduced Serum Medium	ThermoFisher Scientific (Gibco)	31985070	Transfection medium
Pan-flavivirus E protein mAb 4G2	BEI Resources	NR-50327	
Paraformaldehyde	Electron Microscopy Sciences	15710-S	Toxic and carcinogenic
PBS	ThermoFisher Scientific (Gibco)	14190144	
Penicillin/Streptomycin	ThermoFisher Scientific (Gibco)	15140122	
ProLong Gold Antifade Reagent	ThermoFisher Scientific (Invitrogen)	P10144	
Triton-X-100	Sigma-Aldrich	T8787	
Vectastain ABC Kit	Vector Laboratories Inc	PK-4010	Avidin/biotin-based peroxidase kit
Vero Cells	ATCC	CCL-81	
ZIKV E Protein mAb 1176-56	BioFront Technologies	BF-1176-56	
3. Equipment			
Agarose Gel Electrophoresis System	Bio-Rad	1704468	Other commercial sources are acceptable
Class II Biosafety CO2 Cabinet	Varies	N/A	Other commercial sources are acceptable
Desktop Refrigrated Centrifuge	Varies	N/A	
Fluorescence Microscope	Varies	N/A	
High-Speed Refrigrated Centrifuge	Varies	N/A	
MicroPulser Electroporator	Bio-Rad	1652100	Other machines are acceptable
SimpliAmp Thermal Cycler	ThermoFisher Scientific (Applied Biosystems)	A24811	Other machines are acceptable
Vortexer	Varies	N/A	

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Author(s): GINES AVILA-PEREZ, JUN-GYU PARK, AITOR NOGALES, FERNANDO ALMAZAN, LUIS MARTINEZ-SOBRIDO

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Thursday, February 14, 2019

Dear editor,

We have submitted a revised version of our manuscript **"Rescue of recombinant Zika virus from a bacterial artificial chromosome cDNA clone"** by Ávila-Pérez at al. that we would like to be consider for publication at JoVE.

We have carefully considered all the editors and reviewers' comments and suggestions, which have helped us to improve the quality of our manuscript. To address the comments made by the reviewers, as well as to accommodate their suggestions, we have introduced several changes highlighted in *italic and underlined* in the revised version of the document. The following is a point-by-point response to the comments raised by the editorial or the reviewers.

Editorial comments:

Comment: The manuscript will benefit from thorough language revision as there are a number of grammatical errors throughout. Please thoroughly review the manuscript and edit any errors. Some examples:

1) "recent epidemic of Zika virus (ZIKV) highlight…" should be "recent epidemic of Zika virus (ZIKV) highlights"

2) "the importance of establish reverse genetic" should be "the importance of establishing reverse genetic"

Response: We thank the editor for this remark and apologize for the editorial deficiencies. We have thoroughly revised the manuscript in order to correct all the typos and grammatical errors.

Comment: Significant portions show significant overlap with previously published work. Please re-write the text on lines 146-157, 168-170, 176-183, 202-215, 408-409, 497-506 to avoid this overlap.

Response: We thank the editor for this comment and apologize for the overlap with our previous published manuscript. We have included changes in the revised version of the document to reduce the overlap with our previously published work.

Comment: Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Example NOT in imperative voice: step 1.2.

Response: We apologize for this editorial deficiency. Following the recommendation made by the editor, we have revised the manuscript in order to write all the manuscript in imperative voice.

Comment: Split up long steps (e.g. 1.2) so that no more than 4 actions (3-4 sentences) are included per step.

Response: Following the recommendation made by the editor, steps 1.1 and 1.2 have been modified in the revised document to be in imperative voice and to contain less than 4 actions.

Comment: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples: 1) 1.3.7: How exactly? Please describe or cite a reference (only if the step is not to be filmed).

Response: We thank the editor for this comment. A more detailed description on how the integrity of the infectious clone is analyzed has been included in the revised manuscript.

Comment: After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

Response: Following the indications made by the editor, we have highlighted the filmable steps in yellow. Steps 4 and 5 in the protocol will be summarized in the results section of the film.

Comment: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Response: We thank the editor for this remark. Following his/her recommendation, the discussion section has been modified in the revised document.

Comment: Please add scale bars to 4C.

Response: We thank the editor for this suggestion. Accordantly, we have included scale bars in figure 4C in the revised version of the manuscript.

Comment: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are GeneBank, Qiagen QIAEX II Gel Extraction Kit, Qiagen Plasmid Midi, Qiagen Large-Construct, Opti-MEM I, Lipofectamine 2000, etc

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

Response: We apologize for this editorial deficiency. Following the editors suggestions, all the commercial sounding language have been modified in the revised manuscript to generic names.

Comment: If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Response: Figure 1 has been modified and adapted from a previously published manuscript. This has been indicated in the figure legend.

Reviewers comments:

Reviewer #1:

General comment: In this manuscript entitled as "Rescue of recombinant Zika virus from a bacterial artificial Chromosome cDNA clone", the authors described their protocol to rescue an infectious recombinant ZIKV from a full-length cDNA clone assembled in a BAC under control of the CMV immediate early promoter. This protocol provided a powerful method for the generation of flavivirus infectious clones. In general, this protocol is well written and is of interest for virologist.

Response: We appreciate the overall positive and constructive comment made by this reviewer regarding our manuscript of being well written and of interest for virologist.

Comment: toxicity problem of flavivirus cDNA was well accepted. BAC method overcomes this problem without changing of cDNA sequence, which is a big advantage. However, the authors did not mention or compare the yield from BAC method and other methods, since BAC copy number in bacteria is relatively low. Therefore, for rational selection of cloning strategy, I suggest that authors should clearly state this information. Or, at least, discuss this issue more in the introduction section.

Response: We concur with the comment made by the reviewer. Following the recommendation made by the reviewer, we have reference our recently review manuscript by Avila et al. (reference 18), in which the reverse genetics approaches for the generation of recombinant ZIKV are widely discussed, including the rational for the selection of the cloning strategy in the BAC low copy plasmid.

Comment: Together with ref26(Munster, M. et al.), the authors should add another citation describing removal of bacterial promoter from ZIKV cDNA(Zhao, F. et al., Negligible contribution of M2634V substitution to ZIKV pathogenesis in AG6 mice revealed by a bacterial promoter activity reduced infectious clone. Sci Rep. 2018 Jul 12;8:10491).

Response: We agree with the comment made by the reviewer and we thank the reviewer for his/her suggestion. This new reference has been included in the revised version of the manuscript.

Reviewer #2:

General comment: This manuscript describes a protocol for creating, manipulating and rescuing a recombinant Zika virus cDNA clone. Flavivirus clones are well-known for being difficult to work with, being plagued with stability issues, which has hampered research. This problem has been reduced by using a bacterial artificial chromosome, a very low-copy plasmid, to reduce the pathogenic effects of the flavivirus structural genes in the E. coli cells. Using unique restriction sites allows the manipulation of fragments of ZIKV to be placed into the BAC and step-wise create the entire infectious cDNA fragment, including regulatory pieces of DNA like a ribozyme. The final plasmid can be transfected into Vero cells and rescued a few days later by use of the CMV promoter. The protocol also contains details for the visualization of plaques following a plaque assay, which will be helpful for many investigators.

Response: We also appreciate the positive comments on our manuscript made by this reviewer indicating how our manuscript will be helpful for many investigators.

Comment: Line 120. ZIV should be ZIKV?

Response: We apologize for this editorial mistake that has been corrected in the revised document.

Comment: Line 150. Maybe want to clarify the dephosphorylation using CIP, and highlight not to perform this with the insert constructs Z1-Z4.

Response: We concur with the comment made by the reviewer. Following his/her recommendation, a new step describing the dephosphorylation of the vector plasmid using shrimp alkaline phosphatase (SAP) has been included in the revised version of our manuscript.

Comment: 1.3.3. Should the use of a control ligation plate with only the BAC vector be added? It might help to see the differences in vector alone and vector+insert ligations when choosing whether to go forward with the colony growth.

Response: We agree with the comment made by the reviewer. Following his/her suggestion, we have added a note clarifying this point in the revised document.

Comment: Between 1.3.7 and 1.3.8. Add a line saying to choose only one of the correct sequenced BAC-Z1 plasmids to use for the next step. If a novice is reading this and they have 3 clones that are all the same, they will be confused at this step and use all 3. Chose only 1.

Response: We thank the reviewer for this suggestion. Following his/her recommendation, we have clarified this statement from “Pick positives colonies” to “Pick a positive colony” in the revised document.

Comment: Line 288. Change overlaid to overlay.

Response: We apologize for this editorial deficiency and thank the reviewer for his/her remark. This typo has been corrected in the revised manuscript.

Comment: During the growth phases of bacteria, perhaps language about taking the cultures for processing when they're in the log-phase growth only. This can be measured by OD at 600nm by spectrophotometry to quantify late-stage log-phase growth.

Response: We agree with the comment made by the reviewer. Following his/her recommendation, we have added the value of OD_{600} in the revised document.

Comment: Line 516. Dengue is not capitalized.

Response: We apologize for this editorial mistake that have been corrected in the revised manuscript.

Comment: Figure 4A. The calculations are wrong. If one plaque is detected in the first dilution (1/10 of stock), then with an infection of 0.2 ml that would yield a titer of 50 pfu/ml. The readout of 10E1 = 10, which is not measured in this assay, so (1) the axis should reflect MOCK titers as less than the limit of detection for the assay which is 50 pfu/ml, not 10 pfu/ml and (2) the pBAC-ZIKV 0 day post infection reading should only be graphed if it was measured.

Response: We agree with the comment made by the reviewer and apologize for this software mistake. This has been corrected in the revised version of the manuscript.

We hope that by taking into account all the comments and suggestions made by the editor and the reviewers our manuscript has been significantly improved, and we hope that now it could be accepted for publication at JoVE. Finally, we want to thank again the editor and the reviewers for their very helpful and constructive criticisms that have contributed to improve our manuscript.

Sincerely,

Luis Martinez-Sobrido, PhD